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NOROVIRUS REAL TIME RT-PCR DETECTION TECHNOLOGY TRANSITION TO THE JOINT BIOLOGICAL IDENTIFICATION AND DIAGNOSIS SYSTEM (JBAIDS).

5 PROJECT NUMBER

FWH20090192E

6 AUTHORS

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Clinical Research Division
59th Medical Wing, Lackland AFB, Texas

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Enteric Diseases Department
Armed Forces Research Institute for the Medical Sciences
Bangkok, Thailand

Final Report

AFMSA O&M FY10 Project (FWH20090192E):

NOROVIRUS REAL TIME RT-PCR DETECTION TECHNOLOGY TRANSITION TO THE JOINT BIOLOGICAL IDENTIFICATION AND DIAGNOSIS SYSTEM (JBAIDS)

September 21, 2012

Reporting Period: October 1, 2009 to September 21, 2012

Principal Investigator: James C. McAvin (Designated PI 22 July 2011)
Clinical Research Division, 59th Medical Wing, Lackland, AFB Texas

Report Prepared by:

James C. McAvin

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Co-Principal Investigator: COL Carl J. Mason, M.D.
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Distribution Statement

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Project Objectives:

1. Provide scholarly and challenging Graduate Medical Education (GME) opportunities.

Objective 1 Results: GME was not conducted during this O&M FY10 project.

Note: Under AFMSA FY12 - FY13 RDT&E funded projects a formal GME training program has been established by 59th MDW and AFRIMS investigators.

2. Complete RAPID-based test and evaluation data to support AFPMB approval of the RAPID-based *Norovirus* assay for use in disease surveillance using the RAPID.

Objective 2 Results: The primary objective of this project was accomplished. Highly sensitive and specific *Norovirus* genotype I and II (GI and GII) TaqMan assays were developed for direct detection from stool using the "Ruggadized" Advanced Pathogen Identification Device (RAPID). The assays were optimized using Joint Biological Agent Identification and Diagnostic System (JBAIDS) reagents. *Norovirus* GII LoD was established at 50 virus particles per 20 µl reaction volume. Assay sensitivity was 100% and specificity was 100% in testing using a diverse panel of genotypically similar and clinically significant species and strains. The assays are applicable for use with diverse real-time PCR instrumentation to include the JBAIDS.

3. Complete JBAIDS-based test and evaluation data to support JBAIDS program manager approval of the JBAIDS-based *Norovirus* assay for use in environmental (non-human) surveillance using the JBAIDS.

Objective 3 Results: The results of this study support submission of a *Norovirus* JBAIDS Molecular Assay Transition Package to the JPO/JBAIDS program manager for qualification for use in environmental (non-human) surveillance and as a candidate assay for FDA-clearance. Under a separate protocol, the JBAIDS Molecular Assay Transition Package will be formatted as a pre-investigational device exemption (pre-IDE) document. The pre-IDE document will serve as the point of departure for discussion with the FDA Office of In Vitro Diagnostic Device Evaluation and Safety (OIVDES) to obtain guidance and clarification on specific testing requirements for the eventual clearance of the *Norovirus* Detection Kit.

The pre-IDE document will describe the *Norovirus* Detection Kit and its intended use, proposed analytical testing and clinical evaluation strategies. The intent of pre-IDE guidance meetings are to ensure that proposed testing strategy is in line with current OIVDES thinking and is sufficient to support a pre-market notification application. This will be conducted under a separate protocol.

4. Qualified by the JBAIDS program manager as a qualified candidate assay for future research targeted at getting FDA-clearance for human diagnostics on the JBAIDS under a separate research proposal.

Objective 4 Results: The investigational device exemption (IDE) will allow the *Norovirus* Detection Kit to be used in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PMA) application or a Premarket Notification

[510(k)] submission to FDA. Included in FDA OIVDES guidance meetings will be discussion on potential pre-IDE submissions for high throughput systems (HTS) and microarray systems. This work will be accomplished under a separate protocol.

Summary

The fundamental objectives of this AFMSA FY10 O&M study was accomplished; real-time diarrheal disease causative agent detection capability. Highly sensitive and specific *Norovirus* GI and GII TaqMan assays were developed using the RAPID. The assays are applicable for use with diverse real-time PCR instrumentation to include the JBAIDS, HTS, and micro-array based systems.

Project funding was provided by the Air Force Medical Support Agency (AFMSA), Research, Development and Innovations Directorate (SG9), Office of the Surgeon General (AF/SGR) Falls Church, Virginia and the Military Infectious Diseases Research Program (MIDRP), USAMRC, Fort Detrick, Frederick, Maryland. This project was conducted by the Enteric Diseases Department, Armed Forces Research Institute for the Medical Sciences (AFRIMS) and Clinical Research Division (CRD)/59th MDW. This project was jointly funded and executed under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2012. Agreement No.: DODI 4000.19; AFI 25-201).

Products Completed

Norovirus GI and GII TaqMan assays were developed using real-time PCR instrumentation (RAPID). Wet assays were sensitive, specific, and provide rapid, direct detection from stool. Operational applications are FHP disease surveillance and ultimately molecular-based diagnostics. Transfer to the JBAIDS presents no technical challenges. Assay chemistry and formulation allows use on diverse real-time PCR analytic platforms. The *Norovirus* assays are likely candidates for transfer to microarray systems.

Purpose

Noroviruses (NoV), members of the family Caliciviridae, are the principal cause of non-bacterial acute gastroenteritis in children and adults worldwide and are presumed to be as common in military personnel as they are in the general population. Noroviruses cause about 90% of epidemic nonbacterial outbreaks of gastroenteritis around the world and may be responsible for 50% of all foodborne outbreaks of gastroenteritis in the United State (Norovirus: Technical Fact Sheet" National Center for Infectious Diseases;www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-factsheet). Noroviruses most commonly isolated in cases of acute gastroenteritis belong to genogroup I (GI) includes Norwalk virus, Desert Shield virus and Southampton virus, and II (GII), which includes Bristol virus, Lordsdale virus, Toronto virus, Mexico virus, Hawaii virus and Snow Mountain virus.

The genome of NoVs contains three open reading frames (ORFs). ORF1 encodes a large polypeptide containing amino acid sequence motifs observed in many RNA viruses, such as

RNA helicase, 3C-like protease and RNA-dependent RNA polymerase (RdRp). ORF2 encodes the capsid protein with an apparent molecular mass of 58 kDa. ORF3 encodes a small protein likely involves in virion formation. Based on genome sequence analysis, NLV infecting humans can be classified into two genetic groups, genogroup I [GI] and genogroup II [GII]. These two genogroups share less than 60% sequence similarity between groups and approx. 75% similarity within each genogroup. Thus, NoVs comprises many genetically heterogeneous viruses; genogroup I consisting of 14 genotypes and genogroup II consisting of 17 genotypes. Proven real-time polymerase chain reaction (PCR) technologies are currently in use for the detection of Noroviruses. Under this study, we have enhanced the JBAIDS to perform real-time identification and diagnosis by transferring a currently existing *Norovirus* PCR (TaqMan) assays to the JBAIDS platform.

Problem

Non-bacterial acute gastroenteritis is considered as a military significant disease. The ability of medical personnel to accurately diagnose and recognize infectious disease threats in an operational environment is a high priority. The rapid identification of an infectious agent will allow for prompt, appropriate treatment, thereby minimizing morbidity and mortality. Additionally, knowledge about a specific infectious disease threat will allow for the implementation of prevention and control efforts to protect the fighting force.

Results

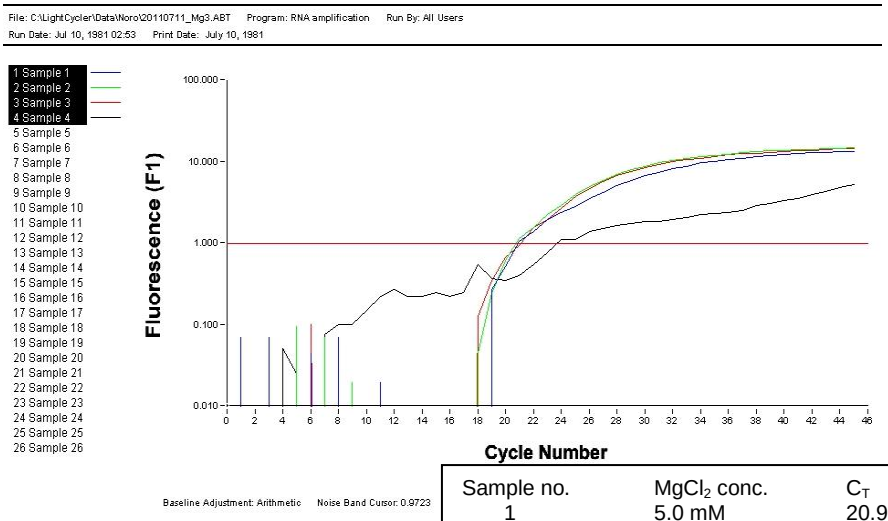
Norovirus Assays Optimization: Idaho Wet Reagent

Norovirus GI and GII assays were separately tested for components optimization using Idaho Technologies wet reagents against synthetic RNA derived from Norovirus GI and GII.

Results:

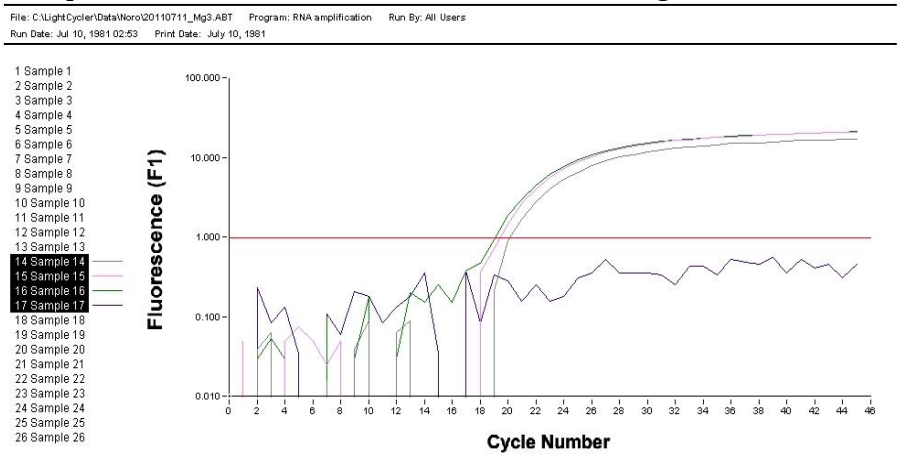
1. Optimum final concentration of $MgCl_2$ was 3.0 mM for both assays (Figure 1 & 2)

Figure 1. Amplification curves of GI reactions at varied $MgCl_2$ concentrations.



Sample no.	$MgCl_2$ conc.	C_T
1	5.0 mM	20.93
2	4.0 mM	20.65
3	3.0 mM	20.29
4	2.0 mM	23.75

Figure 2. Amplification curves of GII reactions at varied $MgCl_2$ concentrations.



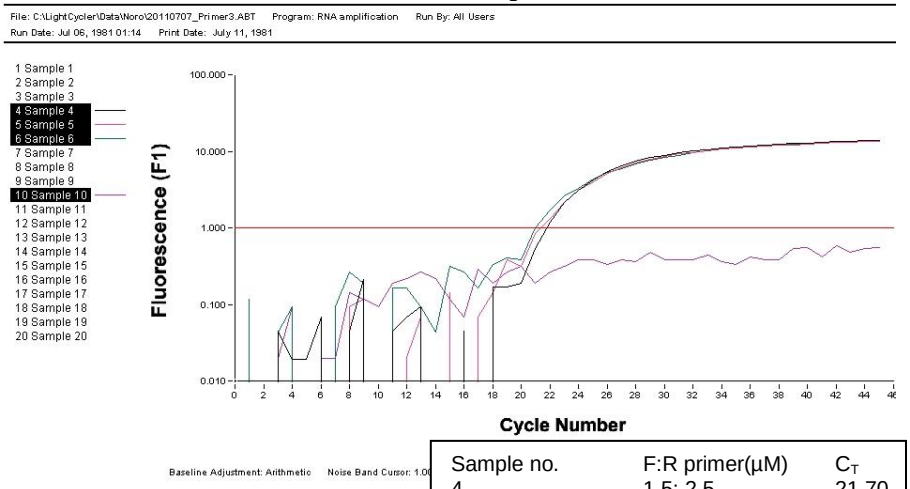
Sample no.	$MgCl_2$ conc.	C_T
14	5.0 mM	19.99
15	4.0 mM	19.38
16	3.0 mM	18.59
17	2.0 mM	neg

2. Optimum final concentration of primers for GI and GII assays was summarized in Table 1. The amplification curves are shown in Figure 3 & 4.

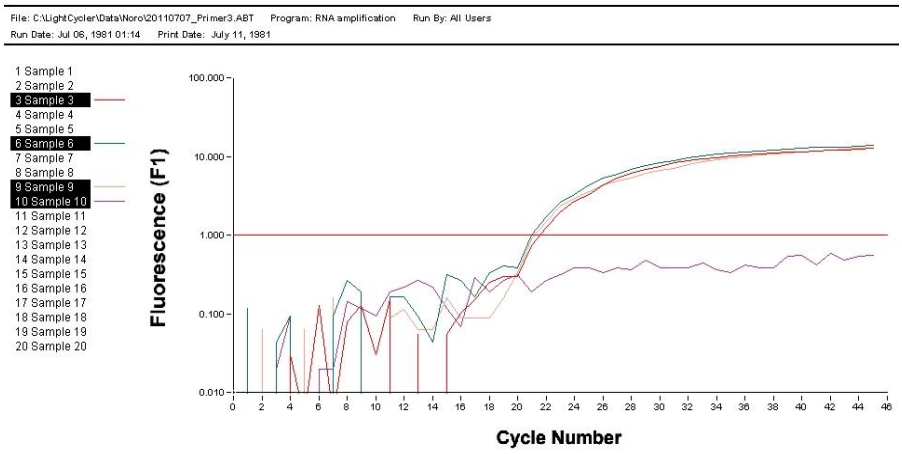
Table 1. The optimum concentration of primers used for Norovirus assays.

Primers	GI assay	GII assay
Forward	3.5 µM	2.5 µM
Reverse	2.5 µM	1.5 µM

Figure 3. Amplification curves of GI reactions at varied primers concentration.

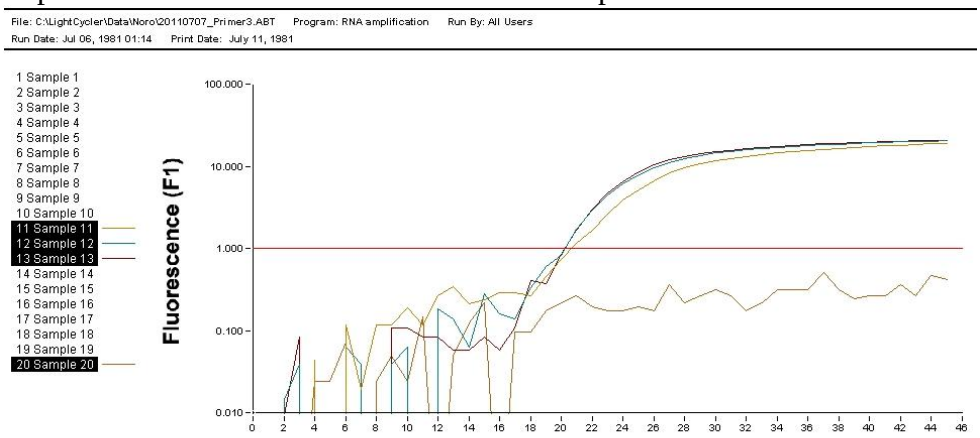


Sample no.	F:R primer(μM)	C_T
4	1.5: 2.5	21.70
5	2.5: 2.5	21.40
6	3.5: 2.5	20.72
10	0.0:0.0	Neg

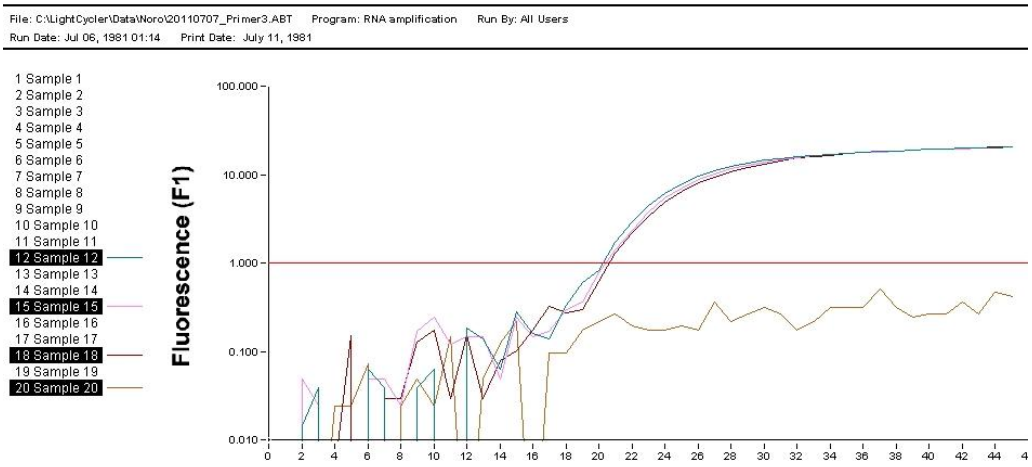


Sample no.	F:R primer(μM)	C_T
3	3.5: 1.5	21.50
6	3.5: 2.5	20.72
9	3.5: 3.5	21.05
10	0.0:0.0	Neg

Figure 4. Amplification curves of GII reactions at varied primers concentration.



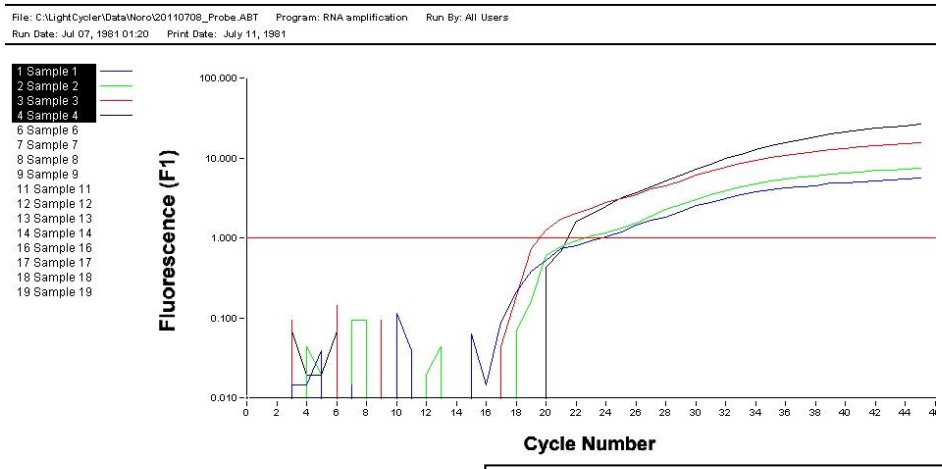
Sample no.	F:R primer(µM)	C_T
11	1.5: 1.5	20.64
12	2.5: 1.5	19.95
13	3.5: 1.5	20.16
20	0.0:0.0	Neg



Sample no	F:R primer(µM)	C_T
12	2.5: 1.5	19.95
15	2.5: 2.5	20.31
18	2.5: 3.5	20.55
20	0.0:0.0	Neg

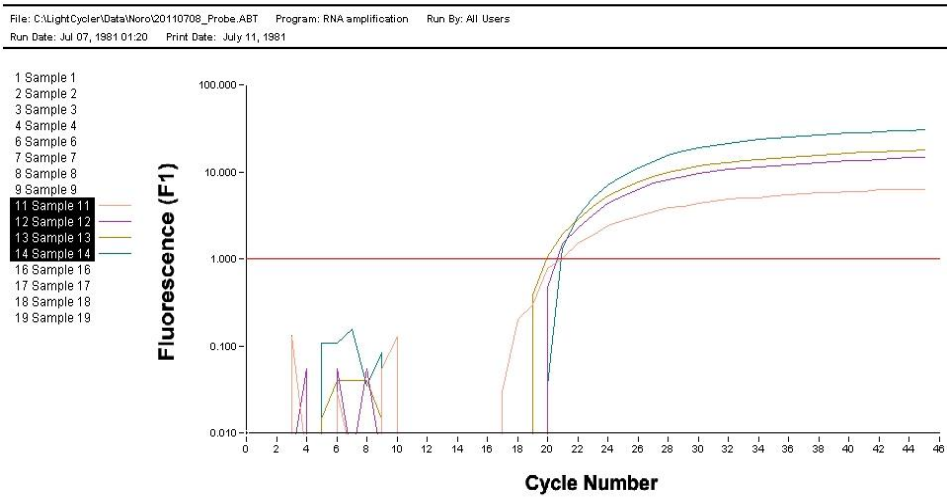
Optimum final concentration of probes was 0.30 µM for both assays (Figure 5 & 6). Gain setting was specified at 8.

Figure 5. Amplification curves of GI reactions at varied probes concentration.



Sample no	Probe (µM)	C_T
1	0.05	24.00
2	0.15	22.50
3	0.30	19.50
4	0.60	21.50

Figure 6. Amplification curves of GII reactions at varied probes concentration



Sample no.	Probe (µM)	C_T
11	0.05	20.99
12	0.15	20.12
13	0.30	19.89
14	0.60	20.67

3. RT-PCR reaction components:

Reagent	GI assay		GII assay	
	Volume (µl)	Final concentration	Volume (µl)	Final concentration
DDW	1.7	-	3.7	-
4X stabilize buffer	5.00	1X	5.00	1X
30mM Mg_2Cl buffer	2.00	3.0 mM	2.00	3.0 mM
20µM forward primer	3.50	3.5 µM	2.50	2.5 µM
20µM reverse primer	2.50	2.5 µM	1.50	1.5 µM
10µM probe	0.60	0.30 µM	0.60	0.30 µM
25 mM dNTPs	0.24	240 µM	0.24	240 µM
1M DDT	0.20	5 mM	0.20	5 mM
200 U/µl RNaseOUT	0.10	1U/µl	0.10	1U/µl
13X Enz diluent	1.68	1X	1.68	1X
20u/µl M-MLV	0.06	1.2 U	0.06	1.2 U
Taq+Ab	0.49	0.1U + 0.221µg/µl	0.49	0.1U + 0.221µg/µl
template	2.00	-	2.00	-
Total	20.00	-	20.00	-

4. RT-PCR reaction conditions:

Reverse Transcription 40°C, 30 min

Heat activation: 94°C, 3 min

Cycling: 94°C 0s; 60°C 20s 45 cycles

Assay optimization: Experiments were performed using GII Norovirus synthetic RNA. This was performed in preparation for the anticipated dry reagents.

1. PCR mixture:

Reagent	GII Volume (µl)	GII Final concentration
DDW	3.7	-
4X stabilize buffer	5.00	1X
30mM Mg2Cl buffer	2.00	3.0 mM
20µM primerF	2.50	2.5 µM
20µM primerR	1.50	1.5 µM
10µM probe	0.60	0.30 µM
25 mM dNTPs	0.24	240 µM
1M DDT	0.20	5 mM
200 U/µl RNase OUT	0.10	1U /µl
13X Enz diluent	1.68	1X
20u/µl M-MLV	0.06	1.2 U
Taq+Ab	0.49	0.1U + 0.221µg /µl
template	2.00	-
Total	20.00	-

2. Cycling conditions:
   Reverse Transcription 40°C 30 min
   Heat activation: 94°C 3 min
   Cycling: 94°C 0s; 55°C 20s 60°C 20s 45 cycles

Assay performance:

Linearity To establish a standard curve, a synthetic RNA of Norovirus GII was used as RNA template. Eight concentrations were prepared ranging from 10^7 copies/reaction to 1 copy/reaction with triplicates for each 10 fold dilution. The standard curve was established from 5 consecutive concentrations. R^2 =-1, Slopes =3.375, Y intercept=34.11

Figure 1. Amplification curves from different template concentrations.

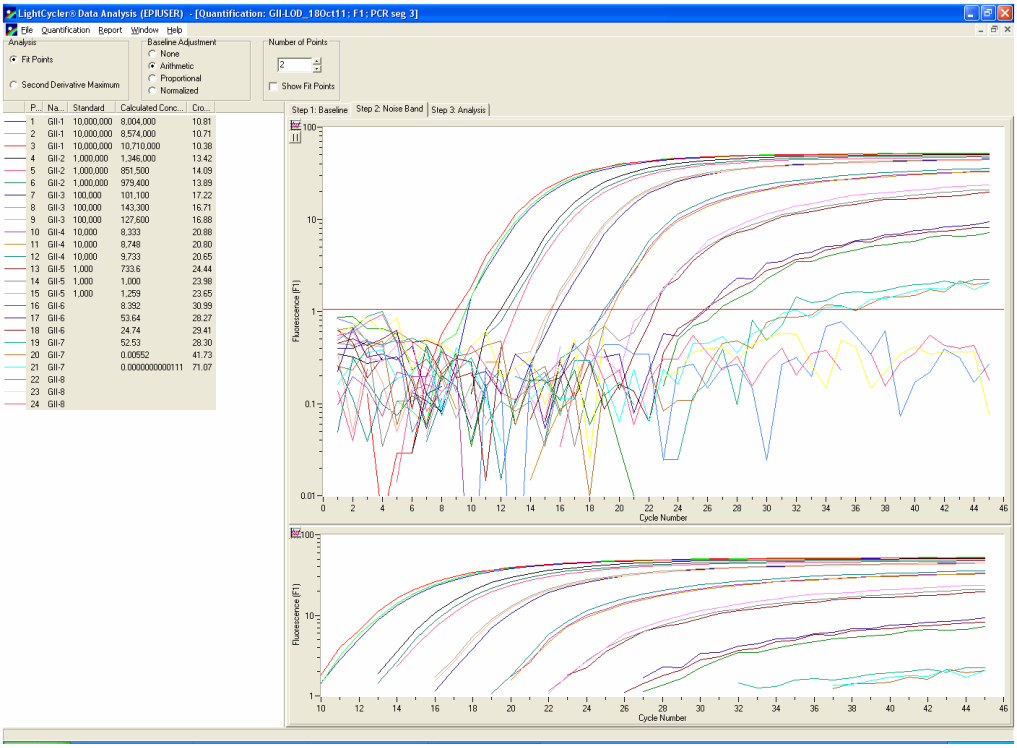
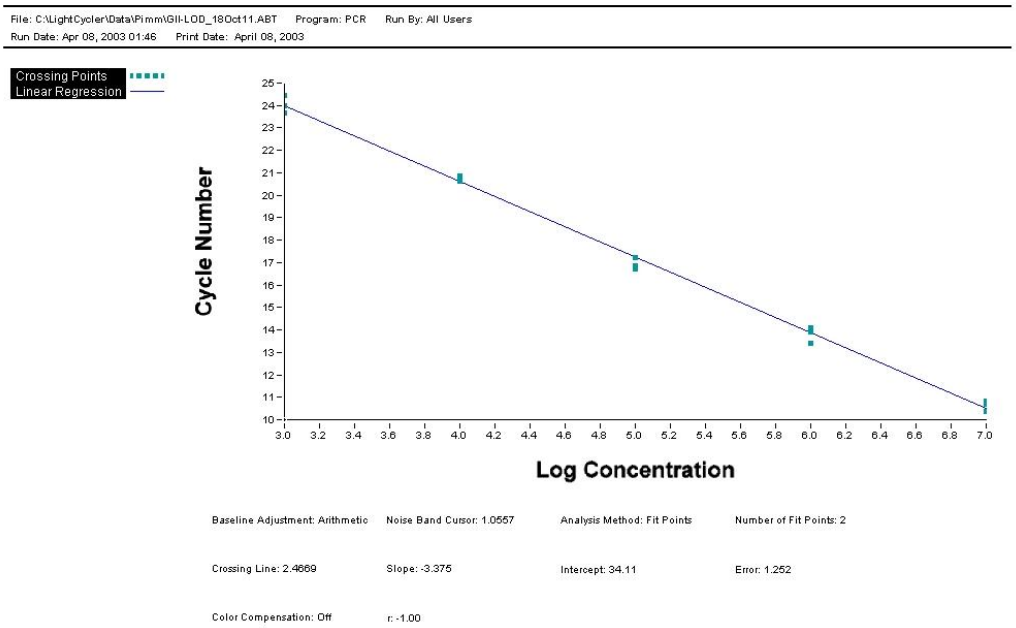
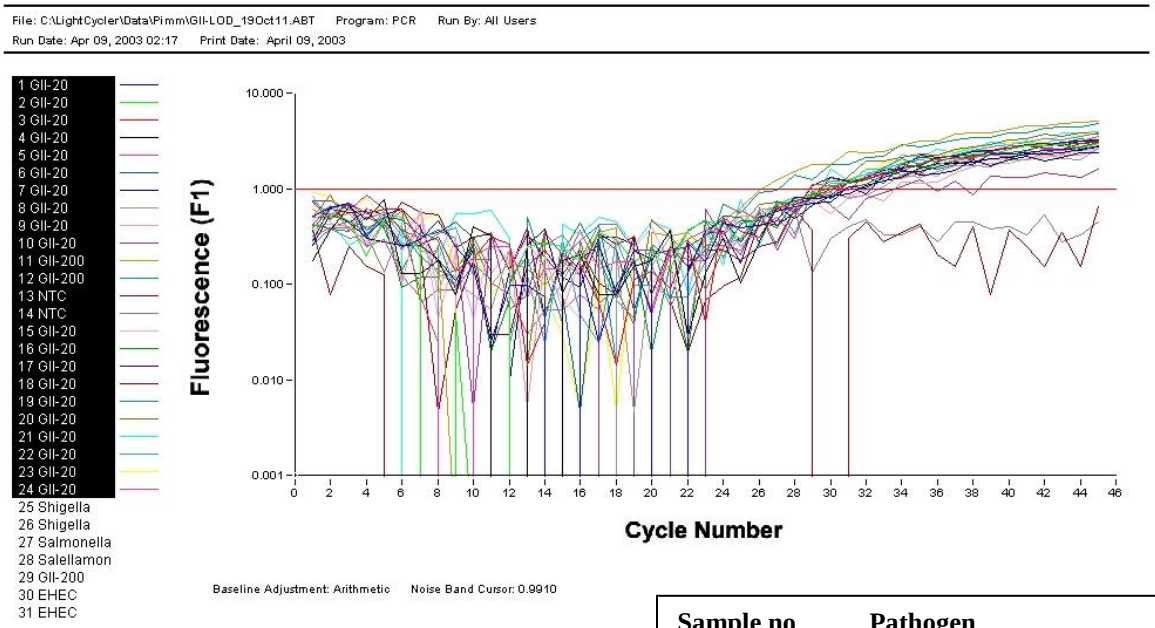


Figure 2. Standard curve showing detection of various template concentrations



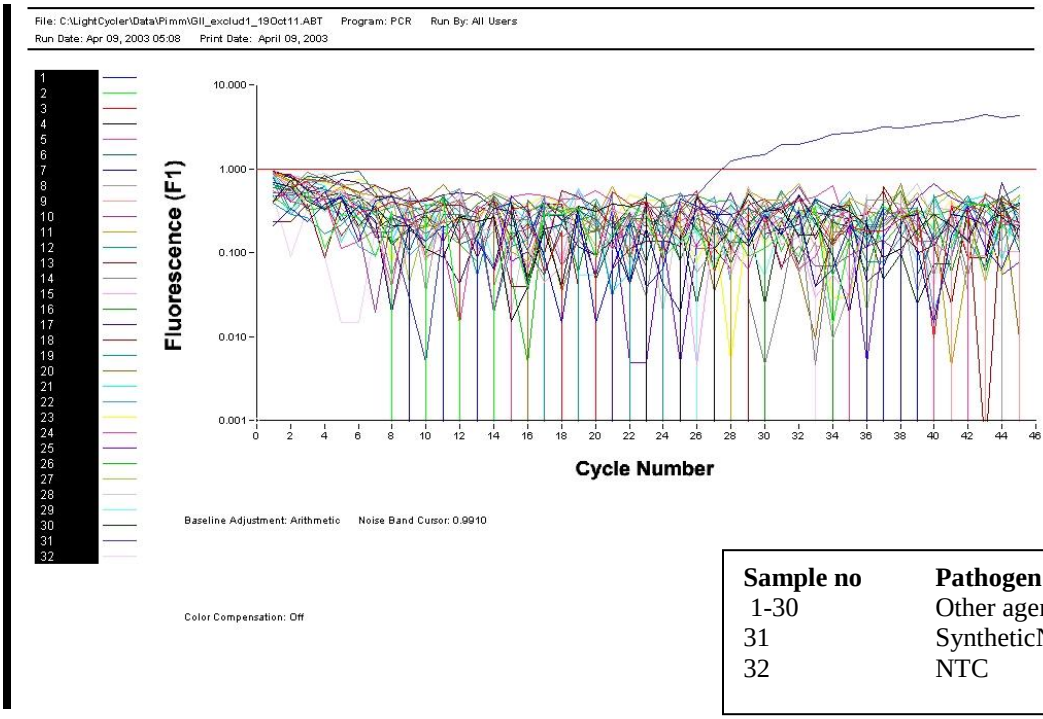
Limit of detection LOD testing were performed with a synthetic RNA of Norovirus GII. A minimum of 58 positive results were required to establish LOD. Sixty replicates were performed at the LOD of the assay consisting of three separate runs of 20 replicates with a minimum of two

instruments utilized (if available), and at least two different operators must be used. The testing will be performed on at least two different days, a minimum of two positive controls and two negative controls will be performed on each run, positive controls 10 fold higher than the concentration tested. LOD of GII assay is 50 copies/ reaction.



Sample no	Pathogen
1-10	50 copies/reaction set 1
11-12	500 copies/reaction
13-14	NTC
15-24	50 copies/reaction set 2

Exclusivity (Test of whether assay cross-reacts with nucleic acids from other organisms):



Sample no	**Pathogen**
1-30	Other agents
31	SyntheticNoV-GII RNA**.**
32	NTC

List of other agents were used in experiment.

Species/Serovar	No. of sample	PCR result
Astrovirus (Known specimen extract)	2	Negative
Campylobacter spp.(Culture)	5	Negative
Citrobacter freundii ATCC8090	1	Negative
Criptospora (Known specimen extract)	2	Negative
Cyclosporidium (Known specimen extract)	2	Negative
E. coli ATCC25922	1	Negative
E. coli (Culture)	2	Negative
E. coli EHEC(Culture)	2	Negative
E. coli ETEC (Culture)	3	Negative
Enterobacter cloacae ATCC23355	1	Negative
Enterobacter aerogenes ATCC13048	1	Negative
Norovirus GI (Synthetic RNA)	1	Negative
Norovirus GI (Known specimen extract)	2	Negative
Proteus vulgaris (Culture)	1	Negative
Salmonella spp.(Culture)	3	Negative
Salmonella typhi (Culture)	2	Negative
*Shigella flexneri*ATCC12022	1	Negative
Shigella sonnei ATCC25931	1	Negative
Vibrio cholera (NAG) (Culture)	2	Negative
Vibrio cholera (Ogawa) (Culture)	1	Negative
Vibrio cholera (Inaga) (Culture)	1	Negative
Sapovivus (Known specimen extract)	2	Negative
Rotavirus (Known specimen extract)	2	Negative

Conclusion

Highly sensitive and specific *Norovirus* genotype I and II (GI and GII) TaqMan assays were developed for direct detection from stool using the RAPID. The assays were optimized using JBAIDS reagents. *Norovirus* GII LoD was established at 50 virus particles per 20 µl reaction volume. Assay sensitivity was 100% and specificity was 100% in testing using a diverse panel of genotypically similar and clinically significant species and strains. The assays are applicable for use with diverse real-time PCR instrumentation to include the JBAIDS.

13 SUPLEMENTARY NOTES

This project was conducted under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2012. Agreement No.: DODI 4000.19; AFI 25-201).

14 ABSTRACT

Highly sensitive and specific *Norovirus* genotype I and II (GI and GII) TaqMan assays were developed for direct detection from stool using the "Ruggadized" Advanced Pathogen Identification Device (RAPID). The assays were optimized using JBAIDS reagents. *Norovirus* GII LoD was established at 50 virus particles per 20 µl reaction volume. Assay sensitivity was 100% and specificity was 100% in testing using a diverse panel of genotypically similar and clinically significant species and strains. The assays are applicable for use with diverse real-time PCR instrumentation to include the JBAIDS.

15 SUBJECT TERMS

Diarrheal, Norovirus, Probe, TaqMan

16 SECURITY CLASSIFICATIONS

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